In comparison to ventricular myocytes, there is relatively little information regarding the transport mechanisms underlying the Ca^{2+} transient in atrial cells. In this study, we focus on the contribution of the sarcoplasmic Ca-ATPase (SERCA) and Na/Ca exchanger (NCX) to the removal of Ca^{2+} from the cytosol in atrial myocytes. Isolated rabbit atrial myocytes were loaded with fluo-4-AM and superfused with a Tyrode's solution at room temperature (20 – 24 °C) in a chamber on a Zeiss Pascal LSM5 confocal microscope. Cells were electrically stimulated at 1 Hz via bath electrodes and linescan images obtained by scanning transversely across the cell. Electrically stimulated Ca^{2+} transients were initiated at the periphery of the cell, rising to a peak within 66.5 ± 15.4 ms. The peak of the transient at the center of the cell was delayed and had reduced amplitude compared with the transient at the cell edge. The decay phase of the twitch transient averaged across the cell width was fitted by a single exponential ($k_{twitch} = 1.71 \pm 0.26\ s^{-1}$). Rapid application of 10 mM caffeine to unload the sarcoplasmic reticulum (SR) produced a large transient that decayed with a rate constant (k_{caff}) of 0.19 ± 0.01 s^{-1}. Following the washout of caffeine, twitch Ca^{2+} transients were markedly diminished but recovered to a steady-state within ~20 s. Subsequent rapid application of caffeine in the presence of Ni^{2+} (10 mM) produced a large Ca^{2+} transient that recovered with a rate constant, k_{Ni}, of 0.02 ± 0.004 s^{-1}. SERCA, NCX and slow pathways were calculated to contribute, respectively, 87.4 ± 1.8%, 10.8 ± 1.4% and 1.8 ± 0.5% of the total Ca^{2+} flux. In conclusion, the overall calcium extrusion pattern appears similar to ventricular myocytes.

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Interplay between Calcium Release and Action Potential Alternans in Rabbit Heart

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Cardiac alternans is recognized as a high risk indicator for cardiac arrhythmias, stroke and sudden cardiac death. At the cellular level action potential duration (APD) alternans correlates with alternation in intracellular calcium release and Ca transient amplitude, thus we investigated relationship between sarcoplasmic reticulum (SR) Ca release and instabilities in electrical activity. Experiments were carried out on single rabbit atrial and ventricular cells. Cytosolic Ca transients were monitored simultaneously with membrane currents or APs recorded with the patch clamp technique. Increase in pacing frequency caused Ca alternans that were accompanied by alternans in AP shape. During alternans APD_{50} of every other beat increased by 108 ± 26% (n=5) and 25 ± 8% (n=5) in atrial and ventricle cells, respectively, and large amplitude Ca transients were always accompanied by short APs and vice-versa (discordant alternans). Recorded APs were applied as stimulation command in voltage-clamp mode (AP-clamp) to record membrane currents. During AP-clamp recordings Ca alternans could be elicited, irrespective whether the command voltage consisted of a series of APs of only long APD, only short APD or alternating APD ('APD alternans clamp'). Furthermore, pacing threshold for Ca alternans was independent of APD. In addition Ca alternans were accompanied by an outwardly directed membrane current of alternating amplitude. This current was also recorded under conditions when all K^+ currents were blocked (replacing K^+ with Cs^+ and 5 mM 4-AP), and preliminary data suggest a chloride conductance. The current was $[Ca]_i$-dependent since it was abolished when SR Ca release was eliminated by removing extracellular Ca or blocking L-type channels with nifedipine. Thus we conclude, that Ca alternans induced by high frequency pacing leads to alternans in AP shape in rabbit myocytes due to alternating changes in activity of Ca^{2+}-activated chloride channels.

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Ca Release Unit Heterogeneity and Entrainment of Ca Waves in Cardiac Myocytes

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Calcium (Ca) is a ubiquitous second messenger regulating many biological functions. The elementary events of local Ca signaling are Ca sparks, which occur randomly in time and space, and integrate to produce global signaling events including intracellular and intercellular Ca waves and whole-cell Ca oscillations. In a recent study using a computational model of Ca signaling in a cardiac myocyte and then experimentally in mouse ventricular myocytes, we demonstrated that criticality is the underlying theoretical mechanism that governs the transition from local Ca sparks to global Ca waves, analogous to a second-order phase transition in thermodynamics. Theoretically, criticality predicts that wave initiation sites should occur randomly and uniformly in space. While this has been demonstrated experimentally at relatively low Ca loads, recent experiments suggest that at higher Ca loads certain regions of the cell dominate the wave initiation process, acting as pacemakers to entrain whole cell oscillations. Here we show that the formation of these pacemaking sites is still governed by the theory of criticality, however, heterogeneities of the ryanodine receptor clusters result in different firing frequencies, with the fastest regions tending to entrain the whole cell. We demonstrate that there is a critical size and relative degree of heterogeneity that must be reached for entrainment to occur, with the novel finding that the degree of entrainment depends on the overall Ca load of the cell. Furthermore, we show that the stochastic nature of the ryanodine receptor channel is crucial to the wave generation process.

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Ca Channel Distribution in T-Tubules and Ca Alternans in Cardiac Myocytes

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In cardiac myocytes, the elementary Ca cycling events are Ca sparks, spatially discrete Ca release events due to random and collective openings of ryanodine receptor (RyR) channels clustered in close proximity to L-type Ca channels (LCCs), forming what are known as Ca release units (CRUs). A typical cardiac myocyte includes about 10,000 to 20,000 CRUs. It is well known that heart failure (HF) remodeling induces changes in whole cell currents and Ca cycling proteins that promote Ca alternans, manifested clinically as pulsus alternans. In addition to electrical remodeling, HF also induces structural remodeling of t-tubules that alters the spatial distribution of CRUs in a myocyte, creating orphaned RyRs that are not associated with LCCs. An interesting question is whether such modifications in the spatial organization of LCCs have independent effects on Ca alternans, even if the whole cell LCC current remains unchanged. Here we address this question by studying the role of the spatial organization of LCCs in the genesis of Ca alternans in a 3D computer model of a ventricular myocyte containing a diffusively coupled network of 20,000 (100x20x10) CRUs. We show Ca alternans is strongly promoted by increasing nonuniformity in LCC distribution among CRUs (simulating T-tubule disruption/dysregulation), independent of changes in the whole cell Ca current. This observation may provide a mechanistic link between T-tubule disruption and Ca alternans observed in failing myocytes. More generally, our results indicate that subcellular details of ion channel distribution can have profound effects on global cellular function not captured by whole cell current measurements.

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Periodic Calcium Waves in Cardiac Myocytes Enhance Susceptibility to Arrhythmia Triggers

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Intracellular calcium (Ca) waves in cardiac myocytes can cause delayed afterdepolarizations (DADs), which are known triggers of cardiac arrhythmias. How these Ca waves are modulated by diffusive Ca-mediated coupling among Ca release units (CRUs) and promote DADs is not fully understood. Here, we hypothesized that myocytes are most susceptible to DADs due to periodic Ca wave activity at intermediate levels of Ca overload. To test this hypothesis, we strengthened CRU coupling by progressively raising intracellular free [Ca] and studied the transition from Ca sparks to waves using both confocal Ca imaging experiments in permeabilized mouse ventricular myocytes and computer simulations of a homogeneous 3D array (100x20x10) of diffusively-coupled CRUs. As free Ca was increased in experiments from 100 nM to 1,500 nM, intracellular Ca release activity evolved through four stages: Stage 1- random sparks and macrosparks arising from multiple sites; Stage 2- irregular aborted Ca waves arising from multiple sites; Stage 3- periodic full Ca waves arising from a small number of sites mostly near cell borders; Stage 4- high frequency ''fibrillatory'' Ca waves exhibiting mixed focal and reentrant features. Raising virtual intracellular Ca in computer simulations reproduced Stages 1-3 but not Stage 4, which may require spatial heterogeneities to occur. In both experiments and simulations, Stage 3 produced the largest whole-cell Ca transients and most synchronous Ca release, making this intermediate stage of Ca overload the most likely to generate DADs of sufficient amplitude to trigger arrhythmias. High frequency ''fibrillatory'' waves under severe Ca overload in Stage 4 diminished Ca transient amplitudes and reduced Ca release synchrony. In conclusion, our findings suggest that ventricular myocytes are most susceptible to DADs when intracellular Ca overload is intermediate rather than mild or severe.

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Reperfusion Ca^{2+} Waves in the Intact Heart: A Possible Trigger for the Generation of Reperfusion Arrhythmias

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Abnormal intracellular Ca^{2+} cycling plays a key role in cardiac dysfunction, particularly during the setting of cardiac ischaemia/reperfusion (I/R). At the onset of reperfusion there is an increase in cytosolic Ca^{2+}, which has been usually associated with an uncontrolled influx from the extracellular space. Ca^{2+} mishandling has been in turn related to reperfusion arrhythmias. However, the basic mechanism for these arrhythmias is still unresolved. We performed experiments to test the hypothesis that the increase in cytosolic Ca^{2+} at the onset of reperfusion originates in Ca^{2+} efflux from the sarcoplasmic reticulum (SR) and propagates through the cell as cytosolic Ca^{2+} waves serving as a trigger for the initiation of reperfusion arrhythmias. By using a combination of pulsed local-field fluorescence (PLFF) and laser scanning confocal microscopy in mouse intact hearts loaded with Rhod-2 and/or Mag-Fluo-4 (cytosolic and/or SR Ca^{2+} measurements) or Fluo-4 (Ca^{2+} sparks and waves) and submitted to global I/R (12/30 min), it was found that ischemia evoked an increase in cytosolic and SR Ca^{2+}. This increase was associated with a significant rise in Ca^{2+} sparks relative to preischemia: 2.07 ± 0.33 vs. 1.13 ± 0.20 sp/sec/100µm ($P<0.05$, ANOVA). Reperfusion evoked an increment in cytosolic Ca^{2+} (Ca^{2+} *bump*) that was associated with a significant decrease in SR Ca^{2+} and in Ca^{2+} sparks with respect to ischemia and a significant increase in Ca^{2+} waves relative to ischemia and preischemia: 0.71 ± 0.14 vs. 0.38 ± 0.06 and 0.25 ± 0.04 w/sec/100µm. The results show the first direct evidence of an increase in Ca^{2+} sparks in ischemia that transform in Ca^{2+} waves during reperfusion. These waves may constitute a main trigger of reperfusion arrhythmias. Supported by NIH R01-HL-084487 and PICT/1903 (FONCYT).

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Oxidation of Ryanodine Receptor following Ischemia/Reperfusion Increases the Propensity of Ca Waves during Beta-Adrenergic Receptor Stimulation

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Beta-Adrenergic receptor (beta-AR) stimulation generates the main positive inotropic response in the heart. However, after ischemia/reperfusion (I/R), beta-AR stimulation can generate arrhythmias. I/R also increased intracellular reactive oxygen species (ROS) production. We investigated whether ryanodine receptor (RyR) oxidation by ROS contributes to the transition from positive inotropic to arrythmogenic effect in ventricular myocytes after I/R. Measurements of contractile and electrical activity from ex vivo rabbit hearts revealed that global I/R produces severe tachi-arrhythmias. Ventricular myocytes isolated from ischemic hearts were characterized by increased both SR Ca leak rates and fractional SR Ca release compared to cells from non-ischemic hearts. Furthermore, myocytes from ischemic hearts showed increased ROS production, decreased level of free thiols in RyRs (RyR oxidation), and increased level of oxidized glutathione (GSSG). Pretreatment of myocytes from ischemic hearts with the reducing agent mercaptopropionylglycine attenuated the oxidization of free thiols in RyRs and normalized systolic SR Ca release and diastolic Ca leak. In myocytes from ischemic hearts, isoproterenol (ISO; 10 nM) led to the occurrence of spontaneous Ca waves, whereas in cells from non-ischemic hearts the same dose of ISO only caused a positive inotropic effect. Treatment of myocytes from non-ischemic hearts with H2O2 (0.1 mM) increased SR Ca leak and fractional SR Ca release to similar levels observed in myocytes from ischemic hearts. Moreover, application of ISO (10 nM) to myocytes from non-ischemic hearts pretreated with H2O2 increased the propensity of Ca waves. These results indicate that augmentation of ROS production following I/R causes RyR oxidation. This post-translational modification of the RyR plays a critical role in the transition from positive inotropic to arrhythmogenic effect during beta-AR stimulation.

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Extracellular Cathepsin-L Alters Ca^{2+} Transient Amplitude and Sarcoplasmic Reticulum Ca^{2+} Content in Adult Rat Cardiomyocytes

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Serum levels of Cathepsin-L (CatL), a ubiquitous cysteine protease, are increased in patients with ischaemic heart disease. It is unknown whether CatL could (i) be released by the heart following ischaemia and (ii) contribute to altered sarcoplasmic reticulum (SR)-mediated function in cardiomyocytes during cardiac disease. *Ex vivo* perfused rat hearts underwent 30min global ischaemia with subsequent reperfusion. CatL activity was detected (Z-LR-AMC fluorometric assay) in coronary effluent throughout the 90 min post-reperfusion period. The effect of extracellular CatL on SR-mediated Ca^{2+} release across a range of reported concentrations found in patients with coronary heart disease was also determined. Ventricular rat cardiomyocytes were isolated, loaded with Fura-2AM and incubated (30min) with recombinant CatL/vehicle at 0.68nM(n=23), 2.70nM(n=22) and 5.40nM (n=18). Cardiomyocytes were field-stimulated (0.5Hz) and perfused with a modified Krebs-Henseleit solution containing CatL/vehicle. CatL activity at physiological pH within the perfusate was confirmed. using epifluorescence microscopy, Ca^{2+} transient parameters during field-stimulation and during rapid application of 10mM caffeine were determined. Ca^{2+} transient amplitude was decreased in a concentration-dependent manner by the above concentrations of CatL (Control:100 ± 4.6 (n=67) *vs.*79.8 ± 7.1 (0.68nM), 68.8 ± 4.8 (2.7nM), 42.9 ± 5.2% (5.4nM); $P<0.05$) *via* reduced transient peak $[Ca^{2+}]_i$ (Control:100 ± 4.2 *vs.*80.6 ± 6.5, 73.0 ± 4.3, 53.6 ± 5.0%; $P<0.05$). The *tau* of Ca^{2+} transient decay was prolonged at 2.7 and 5.4nM (Control:0.9 ± 0.1 *vs.* 1.5 ± 0.1, 2.4 ± 0.2s; $P<0.05$), but not at 0.68nM. The caffeine-induced Ca^{2+} transient amplitude was reduced (Control:100 ± 5.1 *vs.* 78.8 ± 5.0, 81.5 ± 5.2, 60.1 ± 8.5%; $P<0.05$) however *tau* was not significantly affected (1.4 ± 0.1 *vs.* 1.3 ± 0.1s, 1.4 ± 0.1, 1.7 ± 0.1, 1.1 ± 0.1; $P>0.05$). This study demonstrates that CatL is released from ischaemic hearts upon reperfusion and depresses SR-mediated Ca^{2+} release in field-stimulated Ca^{2+} transients in a concentration-dependent manner. Extracellular CatL may therefore have the potential to contribute to cardiac dysfunction in patients with heart failure.

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Exploring the Link between Ca^{2+} Signalling and ATP Dynamics in Cardiomyocyte Dysfunction

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In heart failure the progressive deterioration of cardiac function is linked to Ca^{2+} handling abnormalities, altered metabolism and increased cardiomyocyte apoptosis. However the causal versus consequential nature of these linked phenomena remains to be determined. Here we aimed to investigate cellular ATP and Ca^{2+} environments and necrotic and apoptotic cell death in cellular models of Ca^{2+} dysfunction and metabolic perturbation. We constructed new ATP sensors via fusion of a red fluorescent protein (mCherry) with the pH-independent click-beetle luciferase in both mCherry-Luc and Luc-mCherry configurations. Chimeric proteins exhibited high specific activities of the luciferase partner in vitro (approximately 5 cps/amol protein) and we determined linear fluorescence-luminescence relationships ($r2 > 0.88$) following their expression in COS-7 cells. The dynamic range of the Luc-mCherry configuration was determined via exposure of COS-7 cells to manoeuvres that depleted ATP (e.g. 2-DOG, FCCP) and under conditions of elevated glucose availability (> 6g/L extracellular). In HL-1 cardiomyocyte monolayers, we monitored cellular ATP environments following pharmacologic manipulation of the amplitude and temporal patterning of Ca^{2+} signaling (e.g. using ouabain, staurosporine, BAPTA). Ca^{2+} and ATP environments were monitored through the transitions from spontaneously oscillatory syncytia to non-oscillatory phenotypes. Under these conditions, we measured cellular ATP effluxes and susceptibility to apoptosis using in situ DNA fragmentation and caspase activation assays. This approach reconciles specific perturbations in Ca^{2+} handling, the cellular ATP environment and phenotypic outcomes and builds a more complete picture of the events that contribute to the early stages of cellular dysfunction in chronic heart disease.

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Dissecting the Calcium Transient Refractoriness in Mouse Ventricular Myocytes

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The activation cascade of Ca^{2+} induced Ca^{2+} release (CICR) to produce a $[Ca^{2+}]_i$ transient has been well described; on the other hand, mechanisms responsible for its termination and refractoriness are still a matter of debate. It is known that the ryanodine receptor (RyR) state and sarcoplasmic reticulum Ca^{2+} content ($[Ca^{2+}]_{SR}$) are major determinates in the termination and refractoriness of Ca^{2+} sparks at room temperature. The goal of this work was to evaluate $[Ca^{2+}]_i$ transient refractoriness (CaTR) when RyR state and/or $[Ca^{2+}]_{SR}$ was altered by pharmacological means at physiological temperature (36 ± 1°C). CaTR was measured in mouse ventricular myocytes loaded with Fluo-5F and paced at 2Hz (field stimulation or depolarization through patch pipette) followed by an extra-stimuli with a decreased interval (ESI). This enabled us to measure a $[Ca^{2+}]_i$ transient restitution curves. The $[Ca^{2+}]_i$ transient peak recovered exponentially as ESI increased ($\tau \sim 137$ms). L-type Ca^{2+}